

Review

Molecular biology of glutathione peroxidase 4: from genomic structure to developmental expression and neural function

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Abstract

Selenoproteins have been recognized as modulators of brain function and signaling. Phospholipid hydroperoxide glutathione peroxidase (GPx4/PHGPx) is a unique member of the selenium-dependent glutathione peroxidases in mammals with a pivotal role in brain development and function. GPx4 exists as a cytosolic, mitochondrial, and nuclear isoform derived from a single gene. In mice, the GPx4 gene is located on chromosome 10 in close proximity to a functional retrotransposome that is expressed under the control of captured regulatory elements. Elucidation of crystallographic data uncovered structural peculiarities of GPx4 that provide the molecular basis for its unique enzymatic properties and substrate specificity. Monomeric GPx4 is multifunctional: it acts as a reducing enzyme of peroxidized phospholipids and thiols and as a structural protein. Transcriptional regulation of the different GPx4 isoforms requires several isoform-specific *cis*-regulatory sequences and *trans*-activating factors. Cytosolic and mitochondrial GPx4 are the major isoforms exclusively expressed by neurons in the developing brain. In stark contrast, following brain trauma, GPx4 is specifically upregulated in non-neuronal cells, i.e., reactive astrocytes. Molecular approaches to genetic modification in mice have revealed an essential and isoform-specific function for GPx4 in development and disease. Here we review recent findings on GPx4 with emphasis on its molecular structure and function and consider potential mechanisms that underlie neural development and neuropathological conditions.

Keywords: apoptosis; embryogenesis; excitotoxicity; glial cells; oxidative stress; phosphoinositides; selenium.

Introduction

Molecular biology and bioinformatics have recently revealed at least 25 selenoproteins, five of which are glutathione peroxidases (GPxs) (Kryukov et al., 2003; Utomo et al., 2004). All selenoproteins with known enzymatic activity bear a selenocysteine at their active center that is essential for catalytic activity and cannot simply be replaced by a cysteine residue. The first mammalian selenoprotein was identified by Mills (1957) as the 85-kDa classical GPx or GPx1. Subsequently, additional GPx isoforms were uncovered and today the GPx family comprises seven well-characterized members, all of which except two (GPx5 in humans and mice and GPx7) contain a selenocysteine as the catalytically active amino acid. The presence of selenocysteine at the active site confers a more rapid reaction with peroxide substrates, which make peroxidase catalysis much more efficient (Nauser et al., 2006). On the other hand, there is an evolutionary disadvantage for selenocysteine-containing proteins. The amino acid selenocysteine (U), which is required for biosynthesis of mammalian selenoenzymes, does not have a separate codon on the genomic level. Instead, it is encoded by a TGA-opal codon (Castellano et al., 2004), which usually terminates translation (stop codon). However, in mammals premature termination of translation is counteracted by protein binding to the selenocysteine insertion sequence (SECIS), which is localized in the 5'-untranslated region of the mRNA.

GPx4 is a peculiar member of the GPx family because of its structural and functional characteristics. Whereas GPx1, GPx2, and GPx3 act as homo-oligomeric proteins, GPx4 is a 19–22-kDa monomeric protein. First described by Ursini et al. (1982), GPx4 was later comprehensively characterized with respect to its enzymatic properties (Maiorino et al., 1990). GPx4 is unique in its tissue distribution and specificity for reducing agents (Zhang et al., 1989; Brigelius-Flohe, 1999). This enzyme accepts many reducing substrates in addition to glutathione and can react with a wide array of organic and inorganic peroxides. In particular, its ability to directly reduce phospholipid hydroperoxides within biomembranes and lipoproteins makes GPx4 unique among antioxidative enzymes (Thomas et al., 1990; Sattler et al., 1994). Moreover, recent data suggest a role for GPx4 as a structural protein (Ursini et al., 1999) and as a regulator of apoptosis (Nomura et al., 1999), gene expression (Brigelius-Flohe, 2006) and eicosanoid biosynthesis (Weitzel and Wendel, 1993). Whether the catalytic activity of the enzyme is required for all these biological functions remains to be investigated.

Selenoprotein expression is transcriptionally dependent on selenium supply and thus there is competition

between various selenoproteins for the cellular selenium pool (Brigelius-Flohe et al., 2001; Savaskan et al., 2003). Some selenoproteins such as GPx4 and Dio3 are expressed at normal levels, even under selenium restriction, and rank high in the selenoprotein synthesis hierarchy. In contrast, expression of other selenoproteins, such as GPx1, is rather sensitive towards selenium shortage and their expression is quickly downregulated when selenium becomes limiting. This peculiarity of selenoprotein biology suggests that high-ranking selenoproteins may play a more essential role in the organism (Novoselov et al., 2005). Although the underlying mechanism of selenoprotein expression hierarchy is not fully elucidated, mRNA stability has been proposed as a regulation level (Weiss-Sachdev and Sunde, 2001; Muller et al., 2003). In the case of the low-ranking GPx1 it has been shown that nonsense-mediated decay causes degradation of the mRNA molecule under selenium deficiency. For GPx4 the hypothesis that highly ranked selenoproteins are of major biological relevance is supported by functional inactivation of *gpx4* in mice. Stem cell knockout of *gpx4* leads to early embryonic lethality (Imai et al., 2003; Yant et al., 2003), whereas GPx1 knockout mice develop normally and are fully viable (Ho et al., 1997; de Haan et al., 1998). Interestingly, GPx4 shows a unique cellular distribution in brain compared to GPx1 (Ho et al., 1997; Schweizer et al., 2004; Savaskan et al., 2007). During embryonic development, GPx4 is expressed in principal neurons of the brain.

Various aspects of GPx4 research are discussed in the current literature (Brigelius-Flohe, 1999; Kuhn and Borchert, 2002; Imai et al., 2003), but not the structural biology of GPx4 nor its role in the central nervous system. Recent advances in these fields, including elucidation of the crystal structure of various GPx isoforms and novel data on regulation in response to brain injury (Borchert et al., 2006; Savaskan et al., 2007), prompted us to crit-

ically discuss the state of the art in these fields. We also address the role of GPx4 in neuronal signaling and discuss future developments in GPx4 research.

Coding multiplicity of GPx4 isoforms

The completion of the human genome project indicated the presence of a single GPx4 gene mapped to chromosome 19 (Kelner and Montoya, 1998). Including the 3'- and 5'-flanking regions, *gpx4* spans approximately 4 kbp and consists of seven exons and six introns (Figure 1). This genomic organization, including exon-intron boundaries, is well conserved among mammalian species. In mice the GPx4 gene is located in a syntenic region on chromosome 10 (Knopp et al., 1999). In addition to the paralogous GPx4 gene, two retro-transposed pseudogenes have been identified in the murine genome of various strains with different phylogenetic states (Boschan et al., 2002). One of them was mapped to chromosome 10 in proximity to the paralogous gene and is expressed under the control of captured regulatory elements. In contrast to the second retrotransposon, which is heavily mutated, this pseudogene does not contain major mutations and its expression may thus lead to a functional enzyme.

There are three distinct GPx4 isoforms: cytosolic, mitochondrial, and nuclear. Cytosolic GPx4 (c-GPx4, 19 kDa) originates from *gpx4* starting from the second 5'-ATG (c-ATG) of the first exon (Figure 1). In contrast, for mitochondrial GPx4 (m-GPx4), which has a somewhat higher molecular weight, the first 5'-ATG (m-ATG) of the first exon is used as the translation initiation site. The additional sequence of 192 nt between these two ATGs of exon 1 encodes a targeting sequence for mitochondria (Arai et al., 1996). The targeting sequence is cleaved off in the mitochondrion so that both GPx4 isoforms

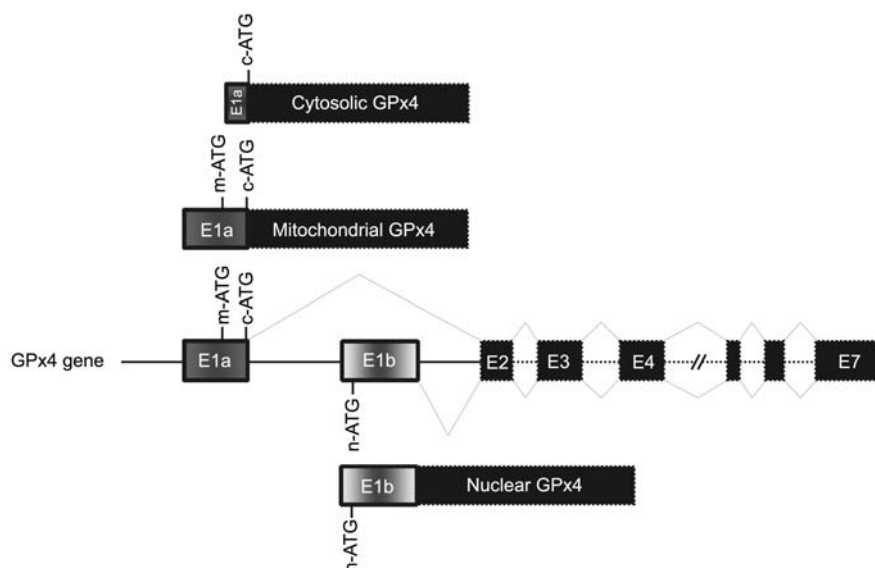


Figure 1 Structure of the *gpx4* gene and encoded isoenzymes.

Exons 2–7 encode for the C-terminal peptide, which is shared by all three GPx4 isoforms. The N-terminal sequences of c- and m-GPx are encoded by the first exon (E1a), whereas the alternative first exon (E1b) encodes the N-terminal part of n-GPx4, including the nuclear import sequence. Boxed areas above and below the genomic scheme indicate the corresponding mRNA. Adapted with modifications from Borchert et al. (2003).

(c-GPx4 and m-GPx4) cannot be differentiated on the basis of their primary structure. This genetic constellation raises the question as to how expression of these two isozymes is controlled. In principle, there are two possible scenarios:

1. Translational mechanism: the major transcriptional product of the GPx4 gene is the m-GPx4 messenger, which is translated to the corresponding protein when the first 5'-AUG serves as the translation initiation site. When the 5'-AUG is functionally silenced, the second 5'-AUG may be recognized as the start codon and the c-GPx4 message will be synthesized. Thus, a switch in isoform specific expression could be explained by functional silencing.
2. Transcriptional mechanism: multiple initiation sites for transcription of the GPx4 gene exist, leading to isoform-specific messages. When a transcriptional initiation site upstream of the 5'-ATG is utilized, an m-GPx4 messenger containing both AUGs (m-AUG and c-AUG) from exon 1 is synthesized. Since the translational machinery usually scans the 5'-UTR for the first available AUG in the mRNA, translation of m-GPx will be initiated. In contrast, when the transcriptional initiation site is located between the first m-ATG and the downstream c-ATG, the resulting message only contains the c-AUG and thus solely the c-GPx4-specific isoform can be translated.

Although the translational mechanisms cannot be completely excluded, there is no evidence of selective silencing of the m-AUG on the translational level. In contrast, several lines of experimental evidence argue for transcriptional mechanisms of isoform-specific expression regulation: (i) multiple mRNA species for GPx4 were found (Pushpa-Rekha et al., 1995; Nam et al., 1997; Knopp et al., 1999; Moreno et al., 2003) and at least one of them starts between the 5'- and the 3'-ATG of the GPx4 gene (Figure 1). (ii) Isoform-specific profiles of GPx4 mRNA species during murine brain development show similar amounts of m-GPx4 and c-GPx4 during early stages of embryonic development (embryonic days E6.5–E15.5). However, in later developmental stages (E16.5) and after birth, the concentration of the m-GPx4 messenger decreased, whereas c-GPx4 mRNA remained unchanged (Borchert et al., 2006). Such mRNA expression kinetics indicates transcriptional regulation. (iii) While in most mammalian tissues expression of the c-GPx4 isoform is predominant, in testis the m-GPx4 messenger is prevalent, suggesting transcriptional regulatory events.

During late spermatogenesis the nuclear isoform (n-GPx4) is predominantly synthesized, which involves transcription of an alternative exon localized in the first intron of the GPx4 gene. This alternative exon (Figure 1) encodes for an Arg-rich nuclear insertion sequence, which is retained with the protein even after nuclear import (Pfeifer et al., 2001). This isoenzyme has an apparent molecular mass of approximately 34 kDa and is specifically expressed in late spermatocytes, but was not detected in most other cells and tissues. The nuclear insertion sequence of n-GPx4 shows a high degree of similarity to other nuclear import sequences, but the mechanism of nuclear import has not been studied in detail so far. It remains unclear what biological role this protein may play in spermatogenesis, since mice lacking this GPx4 isoform develop normally, are fertile and do not show any morphological and functional defects. However, cells isolated from these mice show delayed chromatin condensation and this observation has been related to the sulfhydryl oxidase activity of n-GPx4 (Conrad et al., 2005).

Molecular mechanisms of GPx4 transcription regulation

The molecular mechanisms causing the complex isoform-specific expression patterns of GPx4 in different tissues are not fully understood, but significant progress has been made in this field during recent years. The close proximity of the translational and transcriptional initiation sites for m- and c-GPx4 within exon 1a suggests at least partially overlapping promoter regions and a high degree of interaction between regulatory elements (Figure 2). Functional binding of nuclear factor Y (NF-Y) was initially shown for the proximal promoter region of the human GPx4 gene (Huang et al., 1999). The corresponding binding motif CCAAT is conserved and functional in the murine promoter sequence (Ufer et al., 2003). In addition, functional binding of NF-Y to a second CCAAT element and of stimulating protein 1 (Sp1) to three GC-rich motifs was confirmed in the proximal promoter region of the murine gene (Ufer et al., 2003). This is of particular interest in the context of neurobiology, since these transcription factors are active in developing neurons and reactive astrocytes (Benfante et al., 2005; Bannwarth et al., 2006; Mao et al., 2006). In an attempt to define minimal promoter regions for m- and c-GPx4, various reporter gene assays revealed a complex network of functional interactions between *cis*- and *trans*-regulatory elements (Ufer

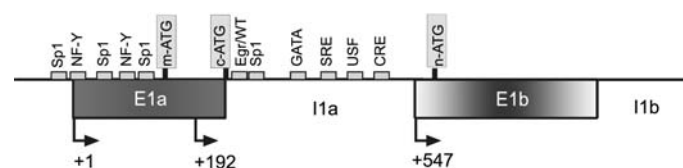


Figure 2 Functional multiplicity in the regulation of the three GPx4 isoforms (m-GPx4, c-GPx4 n-GPx4).

The location of the three major transcriptional start sites (+1 bp, +192 bp and +547 bp) are identified (Nam et al., 1997; Knopp et al., 1999; Moreno et al., 2003). Translation start sites are indicated for each isoform: m-ATG for m-GPx4 (+145 bp), c-ATG for c-GPx4 (+226 bp) and n-ATG for n-GPx4 (+561 bp). Functional *cis*-regulatory elements and their relative positions within the proximal promoter regions are shown as small dark gray boxes. m = mitochondrial; c = cytosolic; n = nuclear.

et al., 2003; Imai et al., 2006). However, the mechanisms that control the differential selection and strength of transcription initiation sites specific to the GPx4 isoforms remain elusive, since neither spatial arrangements of the transcription initiation complex within the GPx4 promoter nor far distance enhancers or silencers have been analyzed so far. Moreover, the mechanisms that are involved in the massive upregulation of GPx4 in spermatogenic cells and in reactive astrocytes in the brain are still not fully understood.

The putative promoter of n-GPx4 is located within intron 1a, in a region where several functional *cis*-regulatory elements (Sp1, Egr1, GATA, SRE, USF and CRE) have been identified (Borchert et al., 2003). In spermatoid cells, CRE was confirmed to bind the transcription factor cAMP-response element modulator (CREM), which is believed to mediate the specific onset of expression for n-GPx4 (Tramer et al., 2004). In somatic cells recombinant CREM expression is able to confer promoter activity to the potential promoter region of intron 1a using reporter gene assays (Tramer et al., 2004). However, in somatic cells, no major promoter activity was found within intron 1a. Instead, this genomic fragment apparently inhibited the proximal promoter activity (located upstream of exon 1a) (Borchert et al., 2003). The mechanistic basis for this apparent inhibition has not been investigated in detail.

Another study reported promoter activity of sequences in exon 1b *in vitro* comparable to the region upstream of exon 1a (Maiorino et al., 2003). Similar promoter activities were identified in the proximal portions of intron 1a, although n-GPx4 expression is more than two orders of magnitude lower than that of c- and m-GPx4 (Maiorino et al., 2003; Imai et al., 2006). Thus, on a quantitative basis it seems to be unlikely that results from the *in vitro* reporter assays adequately mirror the complex regulatory network of isoform-specific GPx4 expression *in vivo*. It is more likely to be an interplay of a variety of *cis*-regulatory elements clustered in the 5'-flanking region of the GPx4 gene (Figure 2). To control isoform-specific GPx4 expression, the pattern of *trans*-acting proteins binding to the 5'-region of *gp*x4 (including the 5'-flanking region, exon 1, intron 1a, and alternative exon 1 and intron 1b) at a given time point is likely to decide which and how strongly a particular GPx4 isoform is expressed. Whether expression of n-GPx4 is driven by a separate promoter within intron 1a *in vivo* and whether additional mechanisms are employed in various tissues remain to be answered. This is of particular interest in light of the recent finding that expression of m-GPx4 and n-GPx4 appeared to be coupled during embryonic development and thus might be controlled by joint regulatory mechanisms (Borchert et al., 2006). In contrast, c-GPx4, which seems to share regulatory elements of transcription with m-GPx4, exhibits different expression kinetics (Borchert et al., 2006).

To comprehensively understand the regulation of GPx4 expression, post-transcriptional events should be considered. Post-transcriptional modifications such as alternative splicing of joint GPx4 mRNA precursors have previously been suggested as factors in GPx4 regulation on the basis of different signaling stimuli or developmental stages (Kuhn and Borchert, 2002; Tramer et al., 2002;

Borchert et al., 2003; Puglisi et al., 2003; Sneddon et al., 2003). Post-transcriptional mechanisms conferred by the 3'-untranslated region that contains the selenocysteine insertion sequence have been studied in more detail. This sequence element is involved in the co-translational incorporation of selenocysteine into the nascent polypeptide chain (Copeland et al., 2000, 2001) and seems to be essential but not exclusive in the regulation of mRNA stability in the case of selenium deficiency (Brigelius-Flohe, 1999; Muller et al., 2003).

Despite these insights, the complex network of tissue- and isoform-specific expression of GPx4 is far from completely understood and more work is needed to determine the molecular interactions of pre-transcriptional, transcriptional and post-transcriptional processes. Moreover, epigenetic regulatory mechanisms of expression, such as DNA and histone modification impacting nucleosome structure, as well as mRNA-protein interaction at the 5'-UTR, have not been studied in detail.

Molecular enzymology and structural aspects of GPx4

GPx4 is a monomeric protein with a molecular mass of approximately 20 kDa that does not undergo major post-translational modification (Roveri et al., 1994). The catalytic selenocysteine residue at position 46 (U46) is essential and mutation of this amino acid to a cysteine (U46C) strongly impairs the catalytic efficiency (Maiorino et al., 1995; Schnurr et al., 2000). Kinetic analysis of the GPx reaction suggested a tert-uni ping pong mechanism, which involves redox shuttling of the selenocysteine (Ursini et al., 1982). The catalytic cycle consists of three consecutive elementary reactions (Figure 3). The first step involves oxidation of the dissociated selenol by the hydroperoxy substrate, yielding a selenenic acid derivative. In the second step, the oxidized enzyme reacts with a thiol group, preferentially reduced glutathione. In the resulting intermediate, the glutathione molecule may be bound covalently with its sulfur to the selenium. The last

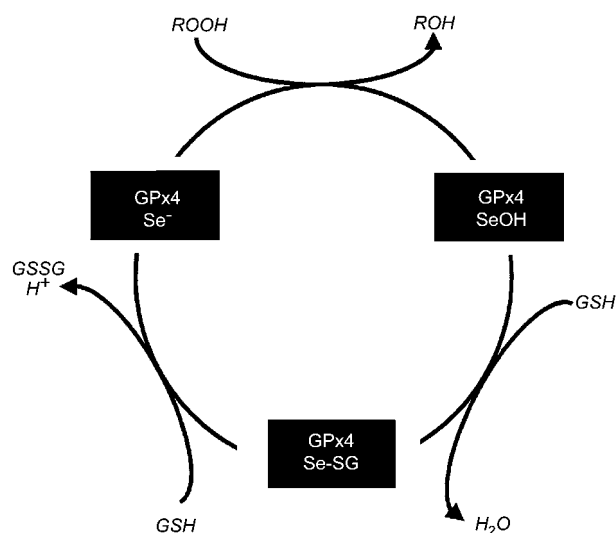


Figure 3 Catalytic cycle of the GPx reaction. Mechanistic details are described in the text.

step of the catalytic cycle involves regeneration of the reduced enzyme by a second glutathione molecule, which splits the transient seleno-disulfide bridge, yielding one oxidized glutathione disulfide molecule. Compared to other GPx isoenzymes, GPx4 exhibits three unique enzymatic and structural properties:

1. The enzyme exhibits broad substrate specificity. In contrast to other GPx isoforms, GPx4 is capable of reducing complex lipid peroxides, even when in highly structured lipid-protein assemblies, such as biomembranes and lipoproteins (Thomas et al., 1990; Sattler et al., 1994; Schnurr et al., 1996).
2. Whereas other GPx4 isoforms prefer reduced glutathione as a hydrogen donor, GPx4 also accepts protein thiols and other reducing equivalents, but not thioredoxin (Maiorino et al., 2007).
3. *In vivo*, GPx4 is usually present as a monomeric protein. However, when expressed in large quantities it exhibits a strong tendency for covalent protein aggregation, and oxidative polymerization of GPx4 has been implicated in the formation of the mitochondrial capsule during spermatogenesis (Maiorino et al., 2005).

Although these functional peculiarities have been demonstrated, their structural basis is not well understood owing to the absence of direct structural data on this enzyme. Structural models of GPx4 have been generated (Aumann et al., 1997; Mauri et al., 2003) based on the X-ray coordinates of other GPx isoforms (Epp et al., 1983; Ren et al., 1997). Although the degree of amino acid conservation among GPx isoforms is rather low, these models served in the past as suitable tools to predict target amino acids for site-directed mutagenesis to impede the catalytic activity (Maiorino et al., 1995, 1998). In retrospect, this hypothetical model adequately mirrored the structural features of GPx4 as indicated by recent crystallographic data (Figure 4). The lack of direct structural information on GPx4 until very recently is partly attributable to the fact that no efficient natural source is known from which the enzyme can be reliably prepared in large quantities. Furthermore, under aerobic conditions GPx4 rapidly undergoes protein polymerization when expressed at higher levels, which severely hampered enzyme crystallization. Thus, to obtain high-quality crystals, expression levels had to be decreased and protein preparation and crystallization had to be carried out under anaerobic conditions. During the past 12 months, five crystal structures for seleno-GPx isoforms and selenium-free enzymes have been deposited in the PDB database (<http://www.rcsb.org/pdb/home/home.do>) including catalytically inactive U-to-G mutants of human GPx1 (PDB entry 2F8A) and GPx4 (PDB entry 2GS3), a U-to-C mutant of GPx2 (PDB entry 2HE3) and GPx5 (PDB entry 2I3Y). Recently we have crystallized the catalytically active U46C mutant of GPx4 at resolution of 1.55 Å (PDB entry 2OBI; Scheerer et al., 2007). The two X-ray data sets for GPx4 (2GS3, 2OBI) confirm that the enzyme is a monomeric protein, in contrast to GPx1, GPx2 and GPx5. In both X-ray structures the affinity tag and the most N-terminal amino acids were not detected in the electron density maps, suggesting a high degree

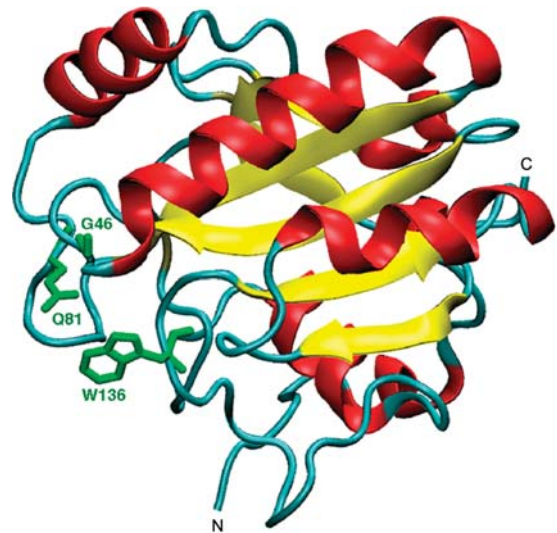


Figure 4 Crystal structures of human GPx4.

The PDB entry 2GS3 was used for structural modeling. The catalytic triad (G46, Q81, W136) is indicated in green. Numbering of amino acid residues is adjusted to the native Sec-containing human GPx4. The four α -helices are in red, and seven β -strands are shown in yellow.

of structural flexibility in this part of the molecule. In general, GPx4 shows the typical thioredoxin motif (Martin, 1995) consisting of four α -helices that are localized at the protein surface and seven β -strands, five of which are clustered to form a central β -sheet (Figure 4). The catalytic triad consists of C46 (corresponding to U46 in the native wild-type enzyme), Q81 and W136. This triad is localized at a flat impression of the protein surface and multiple site-directed mutagenesis of these residues strongly impaired the catalytic activity of the U46C mutant (Figure 4). Q81 and W136 are in hydrogen bond distance to C46 and this arrangement might be important for the catalytic activity. Although structural alignments of GPx4 and GPx1, GPx2 and GPx5 revealed high similarities, GPx4 shows two peculiarities. GPx4 lacks two surface-exposed loop structures (loops 1 and 2) that appear to increase the accessibility of the active site, suggesting a molecular explanation for broad substrate specificity in contrast to other GPx enzymes (Scheerer et al., 2007). Second, surface loop 2 is lacking, which can be considered the structural basis for the monomeric character of GPx4. Thus, recent structural information on GPx4 gives hints as to its unique enzymatic substrate specificity and provides an important basis for the development of small molecule inhibitors.

Developmental regulation and cellular distribution in the brain

Initially, the expression of GPx4 in the central nervous system was indicated by GPx4 activity assays, which suggested low-level expression of the enzyme in the brain compared to testis, liver and adrenal glands (Roveri et al., 1994). In later studies, enzyme enriched from brain homogenate and microsomes was tested and showed approximately the same range of GPx activity as other

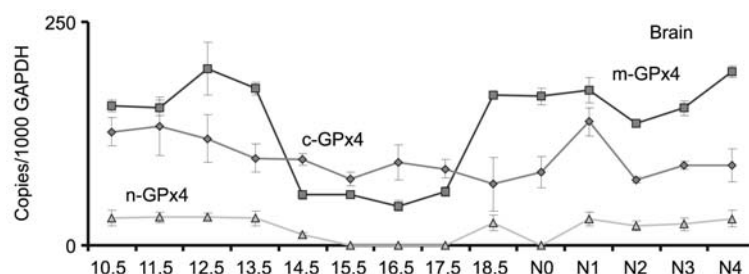


Figure 5 Expression kinetics of the three GPx4 isoforms during brain development.

Expression levels of cytosolic, mitochondrial and nuclear GPx4 mRNAs in murine embryos and postnatal brains (N0–N4) are shown. These data were obtained from real-time RT-PCRs normalized for GAPDH mRNA levels (from Borchert et al., 2006). Note the transient drop and overall low expression of n-GPx4 mRNA.

tissues (Bourre et al., 2000). In brain mitochondria, GPx4 activity was mainly recovered in the inner mitochondrial membrane fraction (Panfili et al., 1991). Direct evidence of GPx4 distribution came from comparative expression analysis, revealing onset of expression as early as during gastrulation (Yant et al., 2003; Borchert et al., 2006). At E6.5, all three isoforms are expressed in extra-embryonic and embryonic structures, including the ecto-, meso- and endoderm. During somite stage development, m- and c-GPx4 mRNA are strongly expressed in headfold, and in particular in forebrain, midbrain and hindbrain and in developing eye (Borchert et al., 2006; Savaskan et al., 2007). Throughout the rostral to caudal neural tube, m- and c-GPx4 transcripts are detectable (Figure 5). Although detected during embryogenesis, the nuclear isoform is only very weakly expressed in neuroepithelium and expression vanished at E16.5 until birth. During postnatal development, GPx4 mRNA is mainly distributed in cortex, hippocampus and cerebellum, indicating a neuronal rather than glial origin. Hence, GPx4 expression peaks at postnatal day 15 and gradually decreases thereafter. In fully mature adult brain, GPx4 is expressed in all neuronal cell layers and most prominently in the hippocampus. Further cellular localization by means of immunocytochemistry identified neurons as a major source of GPx4 in the brain (Figure 6A; Savaskan et al., 2007). Interestingly, GPx4 was recently identified in non-diseased human brain, where it is exclusively expressed by neurons, similar to the situation described for other mam-

malian brains (Figure 6A). Unfortunately, isoform-specific antibodies are still not available and the current antibodies recognize all three GPx4 isoforms (Borchert et al., 2003). Thus, we developed an RT-PCR approach for the detection and quantification of isoform-specific mRNA species (Borchert et al., 2006). We showed that neurons express both c- and m-GPx4 transcripts. Quantitatively, the following expression order has been established: c-GPx4 > m-GPx4 ≫ n-GPx4 (Savaskan et al., 2007). Notably, neuronal cell lines commonly used for analysis of neuronal differentiation and neurite growth and axon collapse (N1E-115, PC12 and HT22) show a similar isoform-specific expression pattern (Figure 6B).

While these data clearly indicate neuronal expression of differential GPx4 isoforms, the functional relevance of the three isoforms remains open. To address this question, various loss-of-function mutants were generated in mice. Genetic disruption of the entire GPx4 gene in the murine genome demonstrated that GPx4 is essential for viability. Homozygous GPx4 knockout mice die *in utero* at midgestation because of gastrulation failure (Imai et al., 2003; Yant et al., 2003). The apparent embryonic lethality has been related to increased apoptosis and cell death leading to malformation of embryonic and extra-embryonic cavities. In contrast, heterozygous GPx4 mice are viable, fertile, and develop normally. However, these mice are more susceptible to γ -irradiation and oxidative stress, pointing to an essential antioxidative function of GPx4 that cannot simply be replaced by other existing

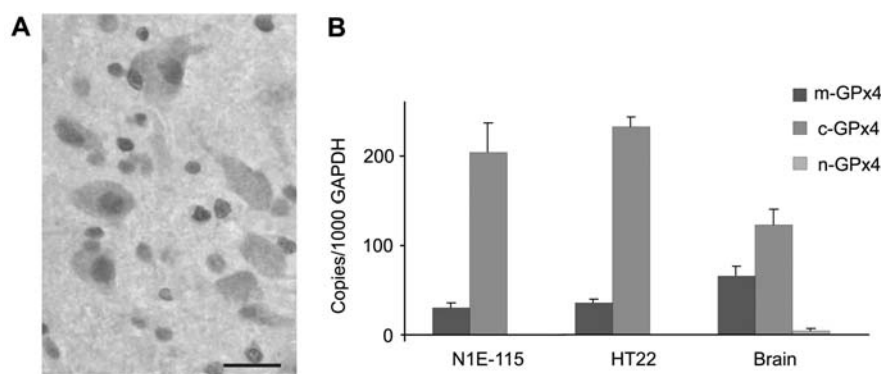


Figure 6 GPx4 is cell-type specifically expressed in human brain.

(A) GPx4 immunoreactive neurons in human cortex (darker grey). Immunopositive neurons are stained and show a typical pyramidal cell shape characteristic for principal neurons (pyramidal cells). The scale bar represents 20 μ m. (B) Isoform-specific expression of GPx4 mRNA in different neuronal cells and in the brain. Note that c-GPx and m-GPx4 are the predominant transcripts in neurons and in total brain. (Taken from Savaskan et al., 2007.)

GPxs *in vivo*. Mice with selective gene inactivation of n-GPx4 are viable without any obvious neuronal failure (Conrad et al., 2005), suggesting that m- and c-GPx4 are required for embryogenesis in general. This is consistent with the isoform-specific expression analysis showing n-GPx4 mainly in testis and solely temporally expressed in the brain (Figure 5). In line with these results, isoform-selective gene silencing using siRNA technology demonstrated that expression of both m- and c-GPx4 is essential for normal brain development, whereas n-GPx4 is dispensable (Borchert et al., 2006). The mechanism by which GPx4 affects neuronal development might be related to regulation of apoptosis and neuronal differentiation. Initial data indeed indicated that knockdown of GPx4 in cultured neurons affects neuronal outgrowth and neurite length (Savaskan and Kühn, unpublished observation). Thus, m- and c-GPx4 are essential enzymes for proper neuronal development and brain function.

An emerging role for GPx4 in neuro-pathological conditions: cell type-specific induction and signaling

Besides the other functions discussed, GPx4 is a multifunctional antioxidant enzyme with anti-apoptotic properties, which relates neuronal expression to cellular anti-oxidative defense. This is especially relevant since neurons, in contrast to glial cells, rely essentially on their GPx activity for clearance of free radicals and lipid peroxides (Dringen et al., 2005). Reactive oxygen species (ROS) are constantly generated in neurons during physiological metabolism. These reactive metabolites must be detoxified and this process is of particular importance for neurons, which are the primary target in degenerative diseases. In fact, increased peroxide formation leads to iron-catalyzed Fenton reactions with hydroxyl radical generation. ROS, in particular hydroxyl radicals, can oxidize lipids, amino-acid side chains and DNA strands, leading to DNA strand breaks and base modifications. Such increases in ROS levels have been found in neuropathological conditions such as trauma, seizures, and ischemia, as well as when neurons are exposed to β -amyloid (A β) peptides (Andersen, 2004). In fact, mice with transgenic overexpression of GPx4 in neurons of the cerebral cortex, hippocampus and in cerebellum are protected from oxidative injury (Ran et al., 2004). More importantly, A β peptide toxicity is decreased and neurons survive upon expression of high levels of GPx4 (Ran et al., 2006). This is of special relevance, since A β toxicity is believed to cause neuronal damage found in Alzheimer's disease (Andersen, 2004). Thus, downregulation of neuronal GPx4 expression may render neurons more prone to deleterious effects of oxidative damage. It has been reported that GPx4 expression decreases H₂O₂ and lipid peroxidation-induced cell death (Ran et al., 2006). Consistent with these results, we recently found that siRNA-mediated knockdown of GPx4 in neurons leads to increased apoptosis and higher susceptibility to oxidative stress (Savaskan et al., 2007). The mechanism of the neuroprotective activity of GPx4 has not been completely clarified. However, it has been shown that siRNA-mediated

knock-down of m- and c-GPx4 in neurons leads to phosphoinositol-bisphosphate (PIP₂) depletion and delocalization from the plasma membrane (Savaskan et al., 2007). This process is an early event in apoptosis at the plasma membrane and appears prior to and independent from caspase-3 activation. Hence, GPx4 prevents extensive cardiolipin oxidation, a mitochondrial phospholipid that in its oxidized state initiates cytochrome c release and caspase-3 activation (Nomura et al., 1999; Petrosillo et al., 2003). GPx4 overexpression does not alter the expression of anti-apoptosis proteins of the Bcl-2 family, such as Bcl-2, Bcl-xL, Bax, and Bad, which would represent the simplest explanation of the beneficial characteristics of GPx4. In contrast, enhanced GPx4 expression could not prevent Fas-induced apoptosis in RBL2H3 leukemia cells, indicating a certain specificity of GPx4 in cell survival (Nomura et al., 1999). These data can be explained by the fact that Fas-induced apoptosis directly activates caspase-8 and does not require any promitochondrial signals (Srinivasan et al., 1998). Thus, GPx4 may specifically impact the signaling of mitochondrial apoptosis. This mechanistic evidence is supported by findings reported on the role of GPx4 during limb development, where expression of GPx4 is restricted to non-apoptotic tissue and manipulation of expression alters apoptosis patterns in tissues (Schnabel et al., 2006). Despite these data linking GPx4 to developmental processes, there appears to be room for GPx4 activity independent of its anti-apoptotic character. The constitutive expression of GPx4 in pyramidal and granule neurons in hippocampus and cortex suggests a more specific cellular role for the enzyme related to neuronal function. Recent data indicate that silenced GPx4 expression in neurons affects plasma membrane PIP₂ assembly and neurite growth and filopodia formation (Savaskan et al., 2007; Savaskan and Kühn, unpublished observation). Thus, GPx4 affects PIP₂-dependent plasma membrane signaling, which represents an event affecting synaptic transmission and other signaling pathways unrelated to caspase activation and induction of cell death. As shown in other tissues, GPx4 might also act as a structural protein in the brain. Thus, expression of catalytically inactive GPx4 enzyme in a GPx4-deficient background would provide one good approach to decipher the underlying functions of GPx4 beyond its catalytic properties.

Recently, c-GPx4 has been shown to be upregulated following brain injury (Savaskan et al., 2007). Interestingly, GPx4 upregulation appears in reactive astrocytes, a glial cell type that does not express this enzyme under normal conditions. Following brain lesion, astrocytes transform their cytoskeleton and migrate towards the lesion, where they are involved in oligodendrocyte repair and myelination, and reconstitution of the blood-brain barrier to prevent neuroinflammation (Bush et al., 1999; Faulkner et al., 2004). Moreover, when astrocytes are genetically inactivated, neuronal regeneration and neurite growth are impaired and demyelination occurs (Blain et al., 2006; Okada et al., 2006). In this respect, GPx4 expression in astrocytes after neurotrauma might be considered as a stress response aimed at neuroprotection to prevent further deterioration. Reactive astrocytes also

show activation of functional *cis*-regulatory elements, the corresponding binding motifs of which are functionally present in the gene promoter region for m- and c-GPx4 (Ufer et al., 2003; Bannwarth et al., 2006; Mao et al., 2006). Thus, astrocytic expression of c-GPx4 can be driven by a transcriptional machinery activated following neurotrauma. However, whether or not GPx4 has additional roles in astrocytic migration or cytoskeletal remodeling requires further analysis.

An intriguing link has been shown for apolipoprotein E receptor 2 (apoER2) expression and GPx4 translation (Andersen et al., 2003). ApoER2 is a member of the lipoprotein receptor gene family and acts as an important regulator of neuronal migration. In particular, apoER2 transduces signaling from the guidance molecule Reelin and provides positional information for developing cortical and cerebellar neurons (D'Arcangelo et al., 1995; Tisir and Goffinet, 2003). ApoER2 deletion thus causes massive brain formation defects, as well as male infertility, which has been related to decreased GPx4 expression (Andersen et al., 2003). Whether apoER2 and Reelin signaling regulate GPx4 expression in the brain and whether GPx4 expression is affected by apoER2 deletion, and hence could cause the phenotype, remain to be demonstrated. A recent report fosters the link between apoER2 and selenoproteins. In their study, Olson et al. (2007) showed that apoER2 functions as a receptor for selenoprotein P (SelP), making it a likely candidate SelP receptor, at least in testis. Whether apoER2 in brain has the same properties and is involved in GPx4 translation has to be tested in the future.

Conclusions and perspective

Recent studies on neuronal expression of GPx4 and the outcome of loss-of-function studies point to an essential role for GPx4 in brain development and neuronal function. As a biocatalyst, GPx4 has been implicated in regulation of redox-sensitive genes, in anti-oxidative defense and in cellular apoptosis, while as a structural protein it is involved in spermatogenesis. Regulation of gene expression of the three GPx4 isoforms is still an ill-defined process that involves transcriptional and post-transcriptional regulatory elements. It is a major task for the future to explore the exact *in vivo* mechanisms of transcriptional regulation, such as chromatin remodeling, DNA and histone modification, regulatory element identification, protein-RNA interactions and others, to put these events into a comprehensive scheme reflecting the whole regulatory network. A second field of intense research is the molecular enzymology of GPx4. The recently identified crystal structures of two GPx4 mutants provide a solid foundation for targeted structure-based protein design. It is now possible to address experimentally detailed questions on GPx reaction (direct structural identification of catalytic intermediates) and to investigate the structural basis for non-covalent oligomer formation and enzymatic peculiarities of GPx4 such as its broad substrate specificity. This may also prompt research on screening for small molecule ligand/inhibitor libraries. A third emerging field of general interest is GPx4 research

in neural development and its role in the central nervous system. In the brain, GPx4 is exclusively expressed by neurons from early development until maturation, when the predominant isoforms are c- and m-GPx4. Hence, following brain trauma, GPx4 is upregulated in reactive astrocytes, a non-neuronal cell type in the brain. Molecular approaches to genetic inactivation and overexpression in mice revealed its essential and isoform-specific function for brain development and its role in oxidative stress and neurotoxicity, which makes GPx4 an ideal target for pharmacological approaches. Since GPx4 is part of the selenoprotein family and has a selenocysteine in its catalytic center, many functions of GPx4 have been attributed to its enzymatic activity. However, it needs to be investigated in the future whether other neurobiological functions such as neurite growth and differentiation are GPx4-dependent and what precise role GPx4 may play in activated astrocytes. Ongoing studies will decipher the isoform-specific signaling in neurons and will explore the essential role of GPx4 in the brain beyond anti-oxidative functions. It will be of major medical relevance to consider potential relations between GPx4 and common neurodegenerative and neoplastic diseases such as Parkinson's disease, Alzheimer disease, and primary brain tumors.

At the time of writing, a study by Burk et al. (2007) was published that reports on slightly lower brain selenium levels in apoER2 knockout mice. Although the question as to whether apoER2 regulates GPx4 expression is still unanswered, these recent data suggest that the apoER2^{-/-} phenotype may also be attributed to overall altered selenoprotein expression in neurons and glial cells.

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